The effect of genome length on ejection forces in bacteriophage lambda

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Abstract

A variety of viruses tightly pack their genetic material into protein capsids that are barely large enough to enclose the genome. In particular, in bacteriophages, forces as high as 60 pN are encountered during packaging and ejection, produced by DNA bending elasticity and self-interactions. The high forces are believed to be important for the ejection process, though the extent of their involvement is not yet clear. As a result, there is a need for quantitative models and experiments that reveal the nature of the forces relevant to DNA ejection. Here we report measurements of the ejection forces for two different mutants of bacteriophage λ, λb221cI26 and λcI60, which differ in genome length by $\sim$30%. As expected for a force-driven ejection mechanism, the osmotic pressure at which DNA release is completely inhibited varies with the genome length: we find inhibition pressures of 15 atm and 25 atm, respectively, values that are in agreement with our theoretical calculations.

1 Introduction

Over the past thirty years, a series of experiments and theoretical work have produced many insights about the importance of internal forces in the bacteriophage life cycle: Early measurements on λ capsids showed that they contained tightly packed DNA (Earnshaw and Harrison, 1977), and subsequent experiments established that DNA packed at these densities exerts a pressure of tens of atmospheres that is dependent on the density and salt conditions (Rau *et al.*, 1984). Any effect of the λ genome length on its life cycle (independent of any particular genes) suggests that internal forces are important, and there are several such effects known: there are upper and lower bounds on the amount of DNA that can be packaged into a λ capsid (Feiss *et al.*, 1977); mutants of λ with long genomes fail to grow without magnesium ions (Arber *et al.*, 1983); and mutants with short genomes fail to infect *pel*$^-$ cells (Katsura, 1983). While magnesium ions reduce the forces between DNA, stabilizing the phage particles, DNA-condensing ions such as putrescine prevent DNA ejection (Katsura, 1983), and osmotic stress can stabilize the genome within phages (Serwer *et al.*, 1983). The evidence seems to indicate that internal forces have an important role in the function of λ: phages with low forces can be incapable of ejecting their DNA forcefully enough to penetrate the cell, while phages with high forces are unable to package their genome or are unstable when fully packaged. A variety of theoretical models of DNA packaging in bacteriophage have been proposed (Riemer and Bloomfield, 1978; Black, 1989; Serwer, 1988; Tzlil *et al.*, 2003; Purohit *et al.*, 2003), but only recently have experiments begun to quantify the forces required to tightly pack DNA into capsids (Smith *et al.*, 2001) and the forces driving DNA ejection (Evilevitch *et al.*, 2003, 2005).

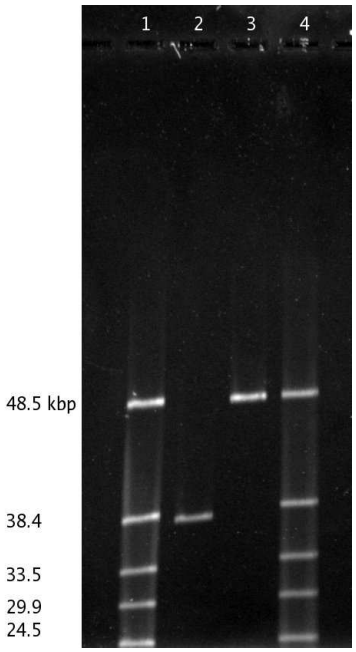

Figure 1: Comparison of λb221 (lane 2) and λcI60 (lane 3) genomes by field-inversion gel electrophoresis. The gel was run with 100/60 V switching for 19h in 0.5x TBE buffer, 1% agarose. Lanes 1 and 4 are λ-mix ladders, with known lengths as shown. Bands contain 0.5–1 ng of DNA, stained with SYBR Gold. The gel shows a single band in lane 2 at 37.9 ± 0.3 kbp and a single band in lane 3 at 48.4 ± 0.3 kbp; both results are consistent with the expected values of 37.7 kbp and 48.5 kbp.

The aim of this paper is to study the effect of genome length on the ejection force of λ DNA, by comparing λcI60, a simple mutant of the wild type with a 48.5 kbp genome, to λb221cI26 (λb221), which has a much shorter genome of 37.7 kbp (Bellett *et al.*, 1971). The reason that measurements with different genome lengths are especially interesting is that simple models of the forces that arise during packaging depend in a precise way upon the genome length. To measure the force, a method reported earlier (Evilevitch *et al.*, 2003) was used: osmotic stress was applied to the outside of the capsids during ejection, halting the ejection at the point where the internal and external forces balance. Using this method, we show that phages with shorter genomes have lower forces, phages with longer genomes eject their DNA with higher forces, and that straightforward theoretical models are sufficient to predict these effects.

2 Materials and Methods

Phages λb221cI26 (λb221) and λcI60 were extracted from single plaques, grown in 3 L cultures of *E. coli* c600 cells, and purified by PEG precipitation, differential sedimentation, and equilibrium CsCl gradients, resulting in $\sim 10^{13}$ infectious particles. After purification, phages were dialyzed twice against a 500-fold greater volume of TM buffer (50 mM Tris, 10 mM $\mathrm{MgSO_4}$, pH 7.4).

To check the genome lengths of the phages used in this experiment, we extracted the DNA with phenol and chloroform from approximately 5×10^9 phages of each type into 500 μL of 0.5x TBE buffer. A quantity of 1 μL (10^7 genomes, or 0.5 ng) was removed from each extraction, mixed with loading dye, heated briefly to 65°C to separate cohesive ends, and pipetted into a 0.5x TBE, 1% agarose gel. A 10 ng quantity of a standard ladder (λ-mix, Fermentas) was included for size comparison. We ran the gel using the method of (Birren *et al.*, 1990), with a simple electrophoresis box (Owl Separation Systems B1A) and a homebuilt voltage inverter, pulsed at 100 V forward, 60 V backward, for 19 h. The gel was stained with SYBR Gold (Molecular Probes) and photographed with an Alphaimager HP (Alpha Innotech). Dust was manually erased from the resulting image, which is shown in Fig. 1. Results were

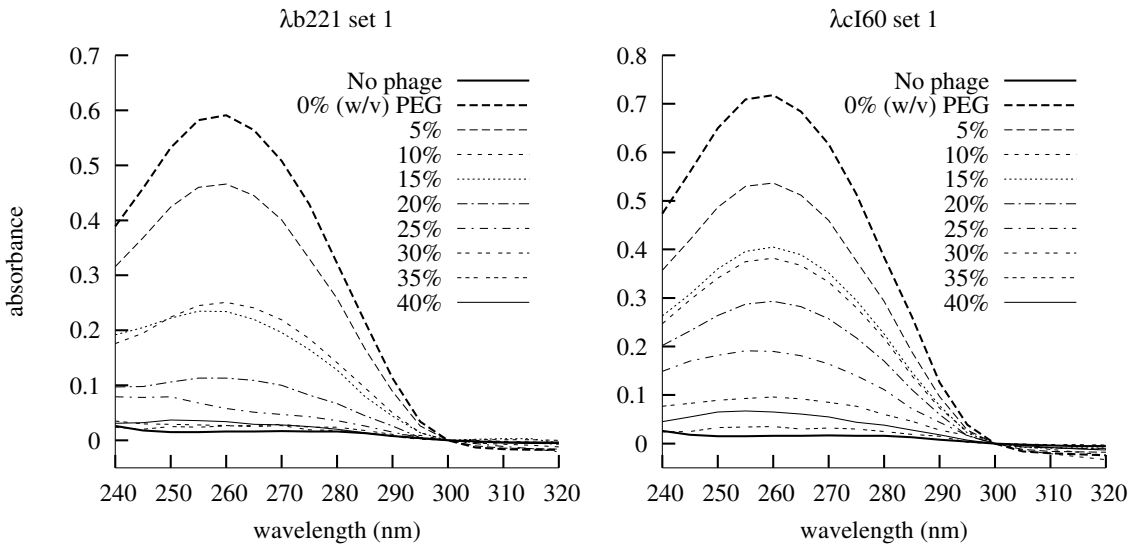

Figure 2: UV absorbance of the supernatant containing fragments of the DNA ejected from λb221 (data set "λb221 set 1", left) and λcI60 (data set "λcI60 set 1", right), aligned at 300 nm. Absorbance curves are shown for various concentrations of PEG, corresponding to different osmotic pressures, and for tests done in the absence of LamB. A second data set taken for each phage is not shown.

consistent with the expected 37.7 kbp genome for λb221 and 48.5 kbp genome for λcI60.

The λ receptor LamB (maltoporin), required to trigger ejection, was extracted from the membranes of *E. coli* pop154 cells. These cells express a *lamB* gene from *S. sonnei* known to be compatible with a variety of λ strains, allowing ejection in the absence of chloroform (Roa and Scandella, 1976; Graff *et al.*, 2002). LamB was affinity-purified in amylose resin and dialyzed twice against TM buffer containing 1% n-octyl-oligo-oxyethylene (oPOE; Alexis Biochemicals #500-002-L005.)

Our method for measuring ejection forces is substantially the same as that described earlier (Evilevitch *et al.*, 2003), with minor refinements that have improved precision. We paid particular attention to the difficulty of pipetting the viscous PEG solutions, trying to minimize systematic and statistical errors that occur when the solution adheres to the pipette tips. A solution of 50% (w/w) polyethylene glycol (PEG) 8000 (Fluka PEG Ultra) was prepared in TM buffer with 0.5% oPOE, and its density was measured at 1.09 g/ml (see also (González-Tello *et al.*, 1994)). This solution was used to prepare solutions of PEG / TM 0.5–1% oPOE at various specified %(w/v) values (see Fig. 2) on an analytical balance. The mass measurements allowed us to set the quantity of PEG in each sample within 0.2 mg, which corresponds to an error in concentration of approximately 0.1% (w/v). Phage solution was added to a final concentration of $\sim 10^{11}$/ml, and DNase I was added at 10 μg/ml. The sample tubes were turned slowly for several minutes to mix the viscous PEG solutions. Purified LamB was added with a wide-mouth tip and the resulting 200 μL solution was mixed quickly by pipetting. A wide-mouth tip is an inaccurate pipetting device, but it is necessary for quickly mixing viscous solutions. To minimize the effect of inaccurate pipetting on the measurements, we used a concentration of LamB that was sufficient for maximal ejection, estimated at ~ 5 μg/ml by UV absorbance. After the addition of LamB, the samples were incubated for 1 h at 37°C, which was sufficient for the reaction to reach its endpoint—complete digestion of the ejected genome fraction by DNase I. Finally, the capsids were separated from the ejected DNA fragments by a centrifugation for 20 h at $18{,}000 \times g$.

After centrifugation, 120 μL of supernatant from each tube was removed to a UV-transparent plastic cuvette (Ocean Optics UVettes) and DNA concentrations were measured with a UV–visible spectrophotometer (LKB Biochrom Ultrospec II). The absorbance curves were aligned at 300 nm (immediately after the DNA absorbance peak) to compensate for absorbance not due to DNA. The resulting curves are shown in Fig. 2. The absorbance values at 260 nm, A_{260}, are linearly related to the amount of DNA ejected from the phage capsids. In contrast with earlier experiments, there was

no measurable background DNA absorbance due to ruptured phage capsids: samples at high PEG concentrations or without LamB had similar values of A_{260} to samples prepared without phages. This is probably because phages were used within one month of dialysis; in contrast, samples of λb221 measured after five months of storage in TM buffer at 4°C had a background $A_{260} \approx 0.1$.

When there is no PEG present, ejection reaches completion (100%), and when no phages are added there is no ejection (0%). Intermediate values were found with a linear interpolation:

$$\text{ejected fraction} = 100\% \cdot (A_{260,\text{with PEG}} - A_{260,\text{no phages}})/(A_{260,\text{no PEG}} - A_{260,\text{no phages}}) \,. \tag{1}$$

An alternative procedure is to use no LamB as a calibration for 0% (Evilevitch *et al.*, 2005).

The weight measurements set $m_{\rm PEG}$, the mass of PEG, and $m_{\rm tot}$, the total mass, for each sample. The % (w/w) weight-weight fraction was computed as $w = m_{\rm PEG}/m_{\rm tot}$. The osmotic pressure at each PEG concentration was then determined with the empirical formula (Michel, 1983)

$$\Pi(\text{atm}) = -1.29G^2T + 140G^2 + 4G \,, \tag{2}$$

where T is the temperature (°C) and $G \equiv w/(100 - w)$. Note that the osmotic pressure is an increasing function of the PEG concentration and a decreasing function of temperature. For this experiment, $T = 37$.

Two complete sets of samples were prepared for each of λcI60 and λb221. Each sample has a statistical error due to weight measurements, pipetting, and spectrophotometry. To minimize systematic effects on the ejected fraction, the two "no phage" and "no PEG" tubes were averaged for each phage. Statistical errors were propagated to yield x and y error bars (see Fig. 3.)

3 Theoretical Model of DNA Packaging

Our theoretical model is based on earlier work (Riemer and Bloomfield, 1978; Tzlil *et al.*, 2003; Purohit *et al.*, 2003, 2005), which describes the packaging energy as a function of the length of DNA in the capsid. We model the λ capsid as a sphere and its genome as a long semiflexible rod. We assume that the rod is wound into a cylindrically symmetric spool (Cerritelli *et al.*, 1997) with local hexagonal packing. The total energy of the packaged DNA can then be approximated by a sum of inter-axial repulsion energy and the bending energy of the rod:

$$E = E_{\rm interaction} + E_{\rm bend} = \sqrt{3}F_0L(c^2 + cd_s)\exp(-d_s/c) + \pi k_{\rm B}T\xi \int_{R_{\rm in}}^{R_{\rm out}} \frac{N(r)}{r} dr \,, \tag{3}$$

where F_0 and c are empirically determined constants describing the interaction between neighboring DNA double-helices, ξ is the persistence length of DNA, L is the length of the DNA within the capsid, d_s is the inter-axial spacing, $R_{\rm out}$ and $R_{\rm in}$ are the radius of the capsid and the inner radius of the DNA spool, respectively, and $N(r)$ is the number of loops of DNA at a distance r from the spool axis. For the persistence length ξ we use 50 nm, though its value in Mg^{2+} buffer may be ∼10% smaller (Hagerman, 1988). The spacing between sequential bases of DNA varies, depending on the base types, from 0.33 to 0.34 nm (Olson *et al.*, 1998). To compute L we disregard this variation and use 0.34 nm times the number of base pairs within the capsid. The inter-axial forces in buffers containing $\rm Mg^{2+}$ have been measured (Rau *et al.*, 1984). Since the values measured for 5 mM and 25 mM $\rm Mg^{2+}$ were not significantly different, we assume that the forces at 10 mM (used in our experiments) will be identical. A least-squares fit to the 5 mM and 25 mM data in (Rau *et al.*, 1984) gives $F_0 = 12{,}000$ pN/nm^2 and $c = 0.30$ nm. The radius of the phage capsid $R_{\rm out}$ is around 29 nm (Earnshaw and Harrison, 1977). Once we know d_s, $R_{\rm in}$, and $N(r)$, we can use Eq. 3 to calculate the internal force on the phage genome as a function of genome length inside the capsid, providing an interpretation of the experimental results.

We calculate the remaining variables as a function of L according to the following recipe, which involves only simple geometrical considerations and elementary calculus. The number of loops $N(r)$ in Eq. 3 is given by $z(r)/d_s$, where $z(r) = \sqrt{R_{\rm out}^2 - r^2}$ is the height of the capsid at distance r from the central axis of the DNA spool. The actual volume $V(R_{\rm in}, R_{\rm out})$ occupied by the DNA spool can be related to the genome length L and the inter-axial spacing d_s to get an expression for $R_{\rm in}$ in terms of d_s, $R_{\rm out}$ and L (Purohit *et al.*, 2003). This expression for $R_{\rm in}$ is substituted

into Eq. 3, which can then be minimized with respect to d_s to give the equilibrium inter-axial spacing as a function of the genome length L inside the capsid. From d_s, $R_{\rm in}$, and $N(r)$, Eq. 3 now gives us the total packing energy as a function of genome length inside the capsid. The internal force $F(L)$ acting on the genome is obtained by taking the derivative of Eq. 3 with respect to L (Purohit *et al.*, 2003).

The preceding construct is a parameter-free model that predicts the ejection force from a λ capsid. Experimental uncertainties in the quantities quoted above should lead to errors of 10–50% in the magnitude of the force predicted, but the shape and relative positions of the curves for different genome lengths should not be strongly affected by these errors (these tests of the parameters are not shown).

DNA ejection in our experiment or *in vivo* is halted at the point where the internal force balances the osmotic force. We have described above how to obtain the internal *force* (and hence, the external osmotic force because of the equilibrium) acting on the genome. But the experimental variable is an osmotic *pressure* (Tzlil *et al.*, 2003). Thus we need to translate this force into a pressure. The force $F(L)$ is given approximately by $\Pi \cdot \pi R_{\rm DNA}^2$, where Π is the osmotic pressure and $R_{\rm DNA}$ is the effective radius of the DNA. We take $R_{\rm DNA}$ as 1.0 nm (bare DNA) plus 0.2 nm, half the PEG monomer length found experimentally (Abbot *et al.*, 1992; Marsh, 2004). This is an exact formula for the osmotic force on a very large area. However, at the PEG concentrations used in our experiment, the diameter of DNA is comparable to the correlation length (mesh size) of PEG, $\sim$1–3 nm (de Gennes, 1979, pp.78–80), so the formula for $F(L)$ is only approximately valid. A scaling expression for the correction could be used (de Vries, 2001; Castelnovo *et al.*, 2003; Evilevitch *et al.*, 2004), but since this result is good only up to a multiplicative constant, its importance is unclear. Hence, to maintain clarity in our analysis we do not use the correction. On the other hand, including this effect could result in a better fit between theory and experiment.

4 Results and Discussion

Fig. 3 shows our experimental results. Partial ejection in both λb221 (37.7 kbp) and λcI60 (48.5 kbp) corresponds closely to that measured earlier in EMBL3 (41.5 kbp): the ejected fraction decreases quickly to 50% at several atmospheres of osmotic pressure, then descends more slowly to 0%. (As shown by Evilevitch *et al.* (2005), this "ejected fraction" corresponds to all phages ejecting the same percentage of their DNA, rather than a percentage of the phages ejecting all of their DNA.) Most importantly, at every pressure where a measurable amount of DNA is ejected, λb221 ejects less DNA than λcI60. Conversely, at any point in the ejection process, the osmotic force on the DNA is always lower in λb221 than in λcI60. For example, the highest forces occur at the point where osmotic pressure completely inhibits the ejection of DNA. This inhibition pressure is in the range 10–15 atm for λb221 and 20–25 atm for λcI60 — a $\sim$100% increase in pressure results from a 30% increase in genome length.

An interesting dip (or bump) in the data is apparent between 1.5 and 3.4 atm. We observed this dip in all data sets collected for this experiment, but its cause is unknown.

Fig. 3 also shows the predictions of our parameter-free theory. Except for the dip, the theory predicts the data quite well. Both the absolute magnitude and the general shape of the curves are predicted correctly. This is a remarkable result because no fitting was done to match the theory to the data. A straightforward inverse-spool model of the DNA arrangement, taking into account only the measured bending elasticity and interaxial forces, correctly predicts the dependence of ejection force on genome length.

Our measurements can be compared with previous results on EMBL3 (Evilevitch *et al.*, 2003), for which the genome length is 41.5 kbp. To do so, however, requires that the weight fractions be corrected in calculating the osmotic pressures, which we have done here for the rest of the data. In this earlier work, the osmotic pressures of the solutions were calculated from the %(w/v) concentrations, without taking into account the density of the PEG solution. Here, we convert it to %(w/w) taking into account the density of the PEG solution. The the density differences can be corrected using the relation between weight fraction and density of PEG solutions (González-Tello *et al.*, 1994). After this correction, we find that the maximum pressure, for example, is 15 atm instead of 19.6 atm. The correction has a smaller effect at lower PEG concentrations; the difference at 10% (w/v) is only 0.2 atm. Fig. 3 shows a comparison between EMBL3 and the mutants used in the present study. Within the experimental uncertainty, the ejection curve for the intermediate length genome lies between those of the 48.5 kbp and 37.7 kbp phages reported here.

An alternative view of the ejection data is shown in Fig. 4. From the ejected fraction p plotted in Fig. 3, we can compute the amount of DNA remaining in the capsid as $N \cdot (1 - p)$, where N is the number of base pairs in the

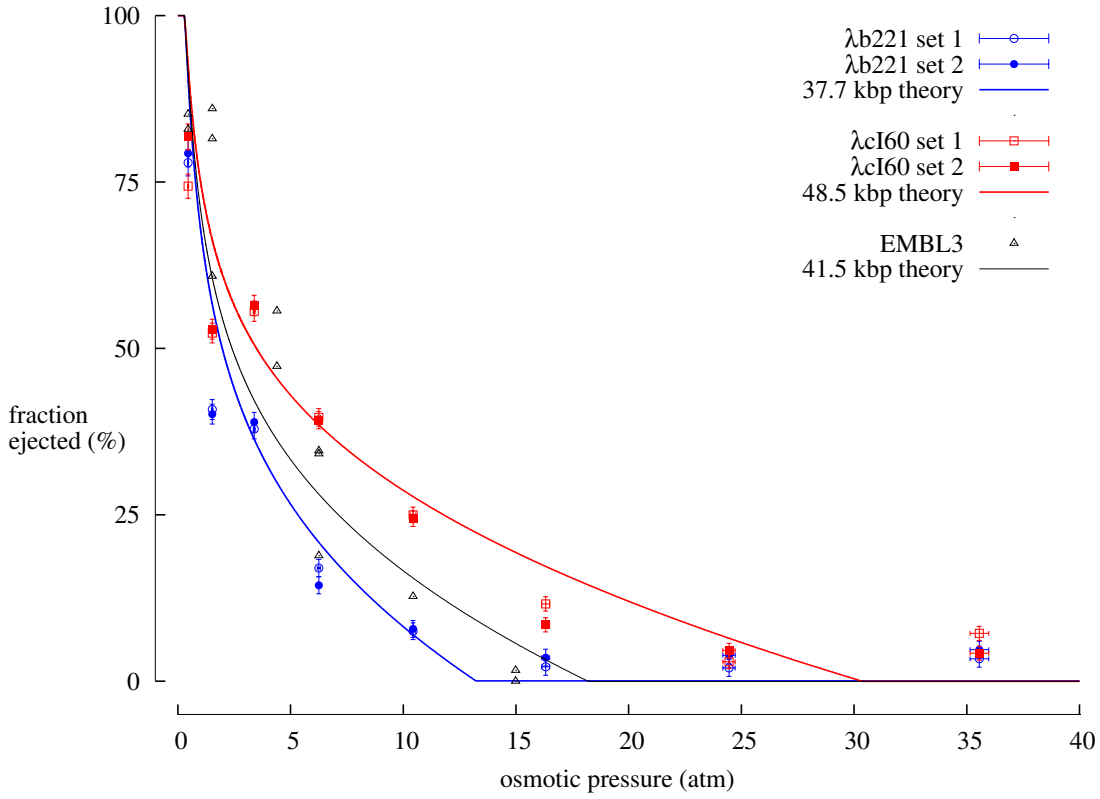

Figure 3: Measured ejection percentages for four sets of data on λcI60 and λb221 (points) compared to theoretical predictions (curves) for 37.7 kbp and 48.5 kbp genomes. Error bars showing one standard deviation were computed from weight measurement, pipetting, and spectrophotometer errors. At all pressures below 15 atm, λcI60 ejects more DNA than λb221. There is an interesting "dip" in the experimental data for both phages, between 1.5 and 3.5 atm, but otherwise the precise trends predicted by theory are seen in the data. The theoretical pressures are calculated with the approximate relation $\Pi\,(\mathrm{atm}) = F/\pi R_{\mathrm{DNA}}^2$, where R_{DNA} is the effective radius of DNA, assumed to be 1.2 nm. Also plotted: data reported earlier on EMBL3 (Evilevitch *et al.*, 2003), with corrections made for a systematic error in the earlier analysis. There is a larger scattering error in the EMBL3 data than in the λcI60 and λb221 data; taking this into consideration, the data sets for all three phages are completely consistent with the expectation that phages with longer genomes eject more DNA at all pressures.

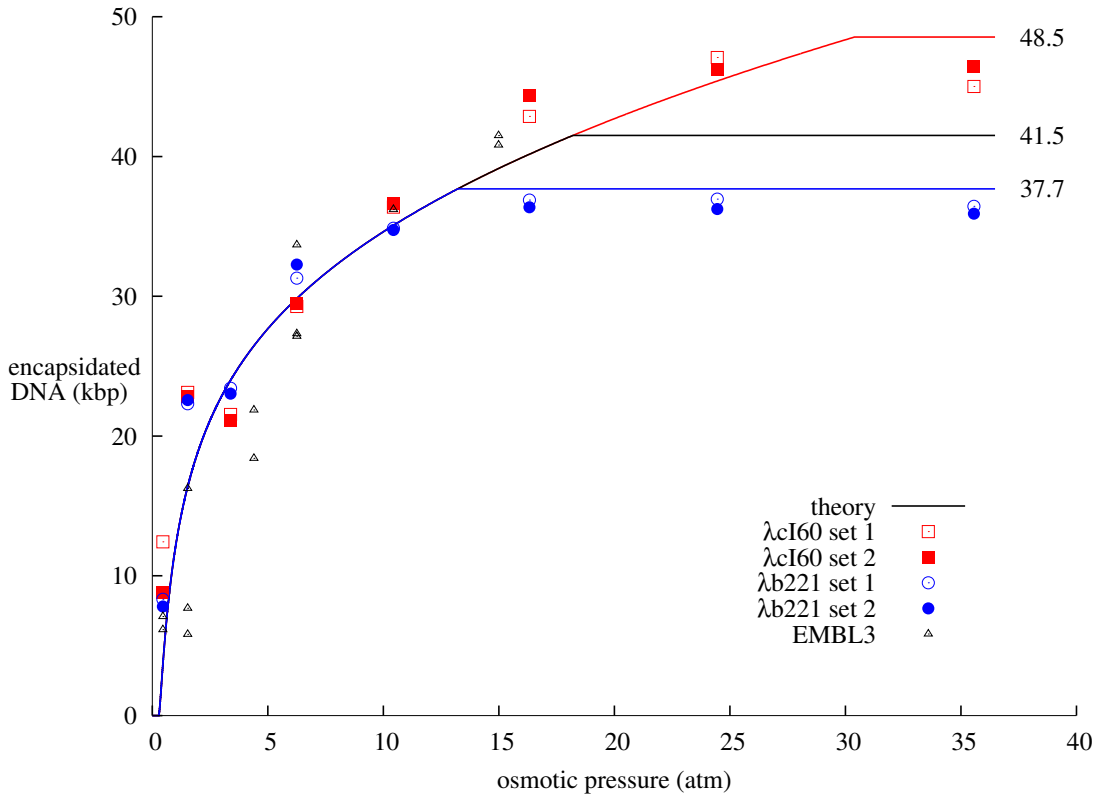

Figure 4: Measured ejection data, plotted as the amount of DNA remaining in the capsid after ejection. The remaining DNA is calculated as $N \cdot (1 - p)$, where N is the number of base pairs in the genome and p is the ejected fraction plotted in Fig. 3. Dotted lines at 48.5, 41.5, and 37.7 kbp correspond to the full genomes of λcI60, λb221, and EMBL3, respectively. As expected, the data points fall on a single curve below 10 atm, then diverge as the maximum pressure is reached for each phage.

genome. Since ejected DNA is digested by DNase I, the amount remaining in the capsid should depend only on the external osmotic pressure, and it should be independent of the genome size of the phage. Indeed, Fig. 4 shows that all of the data below 15 atm falls roughly on a single curve. Above 25 atm, the pressure is sufficient to hold the entire genome within the capsid, so the amount of encapsidated DNA is just equal to the genome length of the phage.

For λcI60, which is almost identical to the wild-type λ, our model and measurements indicate that DNA is ejected with a force of around 10 pN that drops steadily as the genome enters the host cell. What advantage does λ gain from having such a high ejection force? For the phage DNA to enter the *E. coli* cell, it must overcome an internal osmotic pressure of 3 atm (Neidhardt, 1996). Fig. 3 shows that λcI60 can eject roughly 60% of its genome, 30 kbp, against this osmotic pressure. However, the rest of the genome must be actively transported into the cell. Reversible diffusion, in particular, is incapable of transporting the rest of the genome into cell, since the energy barrier that must be overcome is several times $10^3 k_{\mathrm{B}}T$ (Smith *et al.*, 2001; Purohit *et al.*, 2005). A two-step process is implied: first a quick pressure-driven injection of half of the DNA, then a slower protein-driven importation of the remainder (as, for example, by RNA polymerase in phage T7). This two-step process has been observed *in vivo* for the phages T5 (Letellier *et al.*, 2004) and $\phi 29$ (González-Huici *et al.*, 2004), though similar experiments have not been done on phage λ. For λb221, Fig. 3 shows that only 40% of the genome, 15 kbp, is injected in the first step. Since phages with shorter genomes than λcI60 are not infectious, we speculate that 15 kbp is close to the minimum amount of DNA required to initiate the second (non-pressure-driven) stage of importation.

In the case of bacteriophage T7, however, the entire genome is actively transported at a slow, constant speed (Molineux, 2001; Kemp *et al.*, 2004), even though calculations indicate its internal pressure to be similar to that in λ (Purohit *et al.*,

2005). Apparently, something unique to T7 slows down the process (perhaps the observed expulsion of internal proteins from the T7 capsid?), whereas most dsDNA bacteriophages experience a first stage of fast, pressure-driven ejection. Since ejection from λ is much faster (Novick and Baldeschwieler, 1988; Kemp *et al.*, 2004) than from T7, it seems that λ is in this most common category. (As another example, recent experiments by de Frutos *et al.* (2005) and Mangenot *et al.* (2005) show that DNA ejection from bacteriophage T5 proceeds very rapidly between discrete stopping points along the genome. Furthermore, it has been shown in unpublished measurements that T5 ejection can be inhibited by external osmotic pressure.) *In vivo* ejection experiments with λ and *in vitro* experiments with T7 need to be done to explore the differences between these two phages.

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